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The Relative Effectiveness of Human Plasma Glutathione Peroxidase as a Catalyst for the Reduction of Hydroperoxides by Glutathione

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ABSTRACT

To reveal clues to the function of human plasma glutathione peroxidase (GPx), we investigated its catalytic effectiveness with a variety of hydroperoxides. Comparisons of hydroperoxides as substrates for plasma GPx based on the ratio of V_{max}/K_m were blocked by the limited solubility of the organic hydroperoxides, which prevented kinetic saturation of the enzyme at the chosen glutathione concentration. Therefore, we compared the hydroperoxides by the fold increase in the apparent first-order rate constants of their reactions with glutathione owing to catalysis by plasma GPx. The reductions of aromatic and small hydrophobic hydroperoxides (cumene hydroperoxide, t-amyl hydroperoxide, t-butyl hydroperoxide, paramenthane hydroperoxide) were better catalyzed by plasma GPx than were reductions of the more "physiological" substrates (linoleic acid hydroperoxide, hydrogen peroxide, peroxidized plasma lipids, and oxidized cholesterol).

Index Entries: Plasma glutathione peroxidase; extracellular glutathione peroxidase; hydroperoxide; substrate preference; enzyme function.

INTRODUCTION

Mills first described cellular glutathione peroxidase (GPx; EC 1.11.1.9) in erythrocytes, where GPx is the primary means of hydrogen

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peroxide elimination *(1)*. The enzyme utilizes reduced glutathione (GSH) to reduce hydrogen peroxide and organic hydroperoxides (ROOH): ROOH + 2 GSH + ROH + GSSG + H_2O. Peroxide reduction is coupled to the pentose phosphate shunt via NADPH and GSH reductase. GPx protects cells from oxidative damage by removing hydrogen peroxide and lipid hydroperoxides, which can cause lipid peroxidation. Cellular GPx is a tetrameric enzyme with four identical subunits and a mol wt of 84 kDa. Each subunit contains a single selenocysteine residue *(2)*.

GPx activity in blood plasma was initially seen as another form of cellular GPx, which had leaked from tissue. That plasma GPx ("extracellular" GPx) was actually a separate selenoenzyme was first reported by Takahashi and Cohen *(3)*, based on its heat stability and lack of cross-reactivity with antibodies raised against the erythrocyte enzyme. The same group subsequently reported that human plasma GPx was a tetrameric glycoprotein with a mol wt of 100 kDa composed of four identical subunits. Each subunit contained 1 mol of selenium, and the apparent K_m for reduced glutathione was 10 times higher than that of the cellular enzyme *(4)*. Under identical assay conditions, pure human plasma GPx had about ho the specific activity (per mol selenium) of the human erythrocyte enzyme. Cloning and sequencing of the human plasma GPx gene confirmed that plasma GPx was a separate gene product distinct from the cellular enzyme and revealed the presence of an in-frame thymine-guanine-adenine (TGA) selenocysteine codon *(5)*. The deduced amino acid sequence is 44% homologous with the human cellular GPx and includes a "leader" sequence typical of secreted extracellular proteins. 3D models of plasma GPx based on homology with the known X-ray crystallographic structure of cellular GPx imply that the two positively charged arginine residues thought to bind GSH at the surface of the cellular enzyme are not present in plasma GPx, although the immediate chemical environment of selenocysteine in the active site is very similar in the two enzymes *(6)*.

Since plasma GPx lacks a GSH binding site, has only ho the specific activity of cellular GPx, has a 10-fold higher K_m for GSH, and exists in plasma where GSH concentrations are only 1/1000 of intracellular GSH concentrations, it seems ill suited for the reduction of hydroperoxides by GSH in plasma. As a first step in identifying what might be the physiological substrates for plasma GPx, we studied the relative effectiveness of plasma GPx as a catalyst for the reduction of a variety of hydroperoxides by GSH. Plasma GPx's effectiveness as a catalyst for the reduction of hydroperoxides by GSH was assessed by the fold increase in the apparent first-order rate constants that it induced. We focused on the hydroperoxide substrates so we could take advantage of the convenient GSH reductase-coupled enzyme assay for GPx. In this article, we report the relative effectiveness of plasma GPx as a catalyst for the reduction by GSH of hydrogen peroxide, cumene hydroperoxide, t-butyl hydroperoxide, t-amyl hydroperoxide, paramenthane hydroper-

oxide, diisopropylbenzene hydroperoxide, linoleic acid hydroperoxide, methyl-ethylketone peroxide, oxidized plasma lipids, and oxidized cholesterol.

METHODS

Materials

Human blood plasma was stored for 1 d at room temperature in the blood bank and then for 3 d at 4°C in our laboratory Previous reports indicated that the enzyme was stable for several weeks at room temperature *(7).* t-Amy1 hydroperoxide and methylethylketone peroxide were from Witco (Marshall, TX). Paramenthane hydroperoxide and diisopropylbenzene hydroperoxide were from Polysciences Inc. (Warrington, PA). Phenyl-Sepharose ™ CL-4B, DEAE Sephacel™ and Sephacryl S200 HR were purchased from Pharmacia (Alameda, CA). All other chemicals were from Sigma (St. Louis, MO).

Enzyme Activity Assays

Plasma GPx activity was measured by the GSH reductase-coupled method of Paglia and Valentine *(8)* modified to: increase sensitivity with a higher GSH concentration; inhibit any metalloproteins present with EDTA, cyanide and azide; and enhance the solubility of organic hvdroperoxides with Triton X-100 and methanol. One hundred microliters of enzyme preparation were added to 1.5 mL of freshly prepared, ice-cold assay buffer––50 mM Tris-HCl, pH 7.8 (at 37°C), 5.0 mM EDTA, 0.24 mM NADPH, 1 U/mL GSH reductase, 0.01% (v/v) Triton X-100, 10 mM NaCN, 3 mM sodium azide, 1 mM GSH– and preincubated at 37°C for 5 min. Hydroperoxides were added in 50 µL of a methanol/water mixture so that the final assay composition was 1% (v/v) methanol. The assay mixture was transferred to a preheated quartz cuvet in a temperature-controlled spectrophotometer sample holder. The decrease in absorbance at 340 nm was recorded for at least 3 min in a Response II UV-visible spectrophotometer (Ciba Corning Diagnostics, Oberlin, OH). Initial reaction rates were automatically calculated from the linear portions of the absorbance vs time curves. Protein content was determined by the Bradford method *(9)* (Bio-Rad, Hercules, CA), using bovine serum albumin as the standard.

Purification of Human Plasma GPx

Plasma GPx was purified 1290-fold from 1500 mL of human plasma by a modification of the method of Maddipati and Marnett *(7)* (Table 1). We had different chromatography equipment from that used by Maddipati and Marnett, and in our hands at least, the ammonium sulfate precipita-

tion gave a low yield, so we made the following modifications: the 20-50% ammonium sulfate fraction was used instead of 20-30%; DEAE Sephacel™ was substituted for DEAE Sephadex™ A-50; and Sephacryrl™ S-200 HR was used for gel-filtration chromatography instead of Sephadex™ G-200. Aliquots of the purified enzyme were stored at -70°C and thawed just before use each day.

Fold Rate Enhancement Measurements

In preliminary experiments, the dependence of the enzyme-catalyzed reaction rate on hydroperoxide concentration was determined for each hydroperoxide. Saturation of the enzyme with hydroperoxide substrate could not be demonstrated for many of these hydroperoxides, so the reaction rate measurements were made at hydroperoxide concentrations in the ranges where reaction rate increased linearly with hydroperoxide concentration (data not shown). Three rates were measured for each hydroperoxide: the total enzyme catalyzed rate; the uncatalyzed rate without GPx; and the blank rate without GPx or GSH reductase. Each rate was measured in quintuplicate, and means were used for further calculations. Each hydroperoxide's concentration was held constant at either the midpoint of the linear part of its reaction rate vs concentration curve or at its highest achievable concentration. The following empirical equations held under these conditions:

$$\text{Uncatalyzed rate: } R = k \cdot (\text{GSH}) \cdot (\text{ROOH}) \tag{1}$$

The uncatalyzed rates were calculated as the rate in the absence of GPx, minus the blank rate without GPx or GSH reductase. This approach assured that the net NADPH oxidation rate measured was owing only to the oxidation of GSH to GSSG and was not owing to (for example) the direct oxidation of NADPH by ROOH, a reaction of no significance in this context.

$$\text{Catalyzed rate: } R' = k' \cdot (\text{GPx}) \cdot (\text{GSH}) \cdot (\text{ROOH}) \tag{2}$$

The catalyzed rates were calculated as the rate with both GPx and GSH reductase, minus the rate with only GSH reductase. Dividing Eq. 2 by Eq. 1 gives:

$$\text{Catalyzed rate/uncatalalyzed rate} = R'/R = k' \cdot (\text{GPx})/k \tag{3}$$

Rearranging:

$$k'/k = R'/(R \cdot [\text{GPx}]) \tag{4}$$

The ratio k'/k in Eq. 4 (fold rate increase) is the ratio of the apparent rate constant of the catalyzed GSH/ROOH reaction to that of the uncatalyzed

GSH/ROOH reaction, and is a constant for the enzyme with respect to a particular hydroperoxide. Since there would be little physiological need for the enzyme to catalyze a reaction that occurred at a sufficiently fast rate spontaneously (low ratio of k'/k), we reasoned that a larger ratio of k'/k (greater fold rate increase) would indicate which of the many hydroperoxide reduction reactions that plasma GPx can catalyze might be more physiologically relevant.

Preparation of Oxidized Cholesterol

Five grams of cholesterol powder were sprinkled in a thin layer onto the bottom of a 8" x 8" glass baking dish and covered with aluminum foil. Several 1" slits were made in the top of the foil to allow air circulation. The cholesterol was then heated in an oven at 100°C for 8 d. The oxidized cholesterol products were extracted with 35 mL of methanol. The extract was filtered through a glass fiber filter and centrifuged until clear. This solution was used without further purification and stored at -2O°C until used.

Preparation of Oxidized Plasma Lipids

Total plasma lipids were extracted: 423 mL of thawed human plasma were mixed with 1000 mL methanol and 500 mL chloroform in a blender for 2 min. Another 500 mL of chloroform were added and blended for 30 s, and then 500 mL water were added and blended for 30 s. The mixture was allowed to stand at room temperature for 72 h in a graduated cylinder, and the top layer was discarded. The lower chloroform layer was filtered through a Buchner funnel into a round-bottomed flask. Oxygen was bubbled into the chloroform overnight at room temperature, and then continued until all of the chloroform evaporated. The residue was extracted with 5 mL methanol. Butylated hydroxytoluene (0.1%) was added, and the extract was centrifuged and stored frozen at -2O°C until used.

Preparation of Linoleic Acid Hydroperoxide

Linoleic acid (0.1 g), Tween-20 (0.06 g), and sodium borate (0.125 g) were dissolved in 50 mL water, the pH was adjusted to 10.0 with 10 M sodium hydroxide, and the solution was diluted to 66 mL (5.4 mM linoleate). Lipoxidase (735,000 U, Sigma #L3004) was added, and the mixture was bubbled with oxygen and stirred for 40 min at room temperature. HCl was added to a pH of 4.0, and the lipids were extracted with 132 mL of a 2:1 chloroform:methanol mixture. The chloroform layer was washed twice with water and evaporated. The oily residue was dissolved in methanol, centrifuged, and stored at -20°C until used.

RESULTS AND DISCUSSION

Our modification of Maddipati and Marnett's purification methods for plasma GPx did not work as well as their original method *(7)*. SDS-PAGE of the purified plasma GPx revealed that plasma GPx was the predominant protein band, accounting for about 25% of the silver-stained material on the gel (not shown). The presence of protein impurities in our plasma GPx preparations allows the possibility that some of the effects observed were not the result of plasma GPx. However, to our knowledge there have been no reports of other enzymes in human plasma that could catalyze reactions between GSH and hydroperoxides under our assay conditions.

We were not able to use k_{cat} or the ratio of V_{max}/K_m to compare hydroperoxides as substrates for plasma GPx. We found that most of the hydroperoxides were insufficiently soluble to kinetically saturate the enzyme. Although it may have been possible to achieve saturation with these hydroperoxides at lower GSH concentrations, the decreased sensitivity of the assay at lower GSH concentrations precluded this approach. We added 0.01% Triton X-100 and 1% methanol to increase hydroperoxide solubility, but still could not detect any evidence of saturation with many of the hydroperoxides. Since the kinetic relationships of plasma GPx with many of these hydroperoxides had not been previously determined, we believed it inappropriate to apply Michaelis-Menten analysis to these reactions without some evidence of saturation at high hydroperoxide concentrations.

The inability to demonstrate saturation of plasma GPx with hydroperoxides, which prevented application of Michaelis-Menten analysis, was an advantage for applying classical chemical kinetics, since "lack of saturation with hydroperoxide" is just another way of saying "first-order with respect to hydroperoxide." Since the reaction rates were directly proportional to hydroperoxide concentration under our assay conditions, it was possible to write Eqs. 1 and 2 as reasonably accurate rate equations. In fact, since GSH concentration was held constant by continuous rereduction by GSH reductase, the GSH term was superfluous. Therefore, even if the catalyzed reaction had a kinetic order with respect to GSH different from the uncatalyzed reaction, it would not affect our calculations. It is not obvious that the same substrate comparisons would have been obtained with a Michaelis-Menten analysis, since it would not have taken into account the properties of the uncatalyzed reactions and, therefore, would not have included consideration of the physiological benefit of increasing the reaction rate. However, it seems clear that factors that would lead to a lower K_m or a higher V_{max} should also lead to an increase in "fold rate increase." One could argue that the Michaelis-Menten approach has superior physiological relevance because of its inclusion of substrate binding properties and enzyme turnover. However, we believe that since the putative function of plasma GPx is to

Table 1
Purification of Human Plasma GPx

Purification step	Yield (%)	Specific activity (units/mg protein)	Purification (fold)
Plasma	100	0.0029	1
$(NH_4)_2SO_4$ precipitation	26	0.0059	2
Phenyl Sepharose™	2.8	0.641	221
DEAE Sephacel™	3.5	1.75	603
Sephacryl™ S200 HR	3.1	3.74	1290

eliminate toxic hydroperoxides before they decompose to radicals, the more physiologically relevant point is how much more rapidly the enzyme catalyst can dispose of a hydroperoxide compared to the rate of the spontaneous, uncatalyzed reaction between GSH and that hydroperoxide. Ultimately, these two approaches yield complementary data and can both lead to physiologically relevant inferences.

In general, reductions of synthetic hydroperoxides were better catalyzed than reductions of the more "physiological" hydroperoxides (Table 2). The five hydroperoxides whose reductions were best catalyzed by plasma GPx had in common hydrophobic structures of aromatic rings or highly branched compact hydrocarbons. The hydroperoxides whose reductions were least-well-catalyzed by plasma GPx tended to be more polar and more similar to hydroperoxides likely to be encountered physiologically. We concluded that linoleic acid hydroperoxide is a more appropriate substrate for plasma GPx than is hydrogen peroxide, which is more appropriate than oxidized cholesterol. This is consistent with recent work comparing the suitability of hydrogen peroxide, linoleic acid hydroperoxide, and cholesterol hydroperoxide as substrates for plasma GPx based on their individual Dalziel coefficients *(10)*. It is possible, however, that the relatively poor performance of oxidized cholesterol as a substrate in our system was owing, at least in part, to the presence of large amounts of impurities in our oxidized cholesterol. However, our result that the reduction of oxidized plasma lipids is better catalyzed by plasma GPx than the reduction of oxidized cholesterol also agrees with recent reports that plasma GPx can reduce phospholipid-derived hydroperoxides, but cannot reduce cholesterol hydroperoxides *(11)*, even though our substrates were not purified.

Table 2
Fold Rate Enhancement by Plasma GPx of Hydroperoxide Reduction by GSH

Hydroperoxide	Concentration	Rate enhancement *(k´/k)*
Cumene hydroperoxide	0.006 mM	4930
t-amyl hydroperoxide	0.040 mM	2120
t-butyl hydroperoxide	0.009 mM	1580
Paramenthane hydroperoxide	0.040 mM	1420
Diisopropylbenzene hydroperoxide	0.094 mM	1060
Linoleic acid hydroperoxide	0.015 mM	225
Methylethylketone peroxide	0.040 mM	138
Hydrogen peroxide	0.036 mM	116
oxidized plasma lipids	1 - 8 dilution [a]	50
Oxidized cholesterol	l-64 dilution [a]	8

[a] Dilution of methanol extracts. Absolute concentrations were not determined.

Maddipati and Marnett applied Michaelis-Menten kinetics to plasma GPx with both 13-hydroperoxylinoleic acid and hydrogen peroxide at 5 mM GSH, without clear evidence for saturation by the hydroperoxides *(7)*. Their calculation of k_{cat} from Lineweaver-Burk plots implied that hydrogen peroxide was about 2½ times better as a substrate for plasma GPx than 13-hydroperoxylinoleic acid. We found that the rate of reduction of peroxidized linoleic acid was increased twice as much by plasma GPx as was the reduction of hydrogen peroxide, essentially the opposite result. This difference may have been the result of differences between Michaelis-Menten kinetics analysis and the kinetics analysis we used, or it might have been the result of their use of a well-characterized, pure isomer of linoleic hydroperoxide (13-00H), whereas our substrate was not purified. However, it is hard to conceive how the presence of impurities in our linoleic acid hydroperoxide could have increased the rate of its reaction rather than inhibiting the reaction as one might expect.

Given its poor affinity for GSH, its lack of a GSH binding site, and the low concentration of GSH in plasma, if plasma GPx is an oxidation-reduction enzyme, it seems unlikely that its physiological role in plasma would involve catalytic reduction of hydroperoxides by GSH. It has been proposed that plasma GPx might serve as an antioxidant compound rather than an antioxidant catalyst by chemically reducing hydroperoxides in plasma using its preformed, reduced selenocysteine residues *(6).* However, it is difficult to envision how micromolar concentrations of protein-bound selenocysteine could serve as an effective antioxidant compound in plasma.

It is also possible that plasma GPx uses an oxidizable compound in plasma other than GSH to provide reducing equivalents. An intriguing possibility is suggested by a report that the dithiol enzymes glutaredoxin (thioltransferase) and protein disulfide isomerase both catalyze the reduction of dehydroascorbic acid by GSH *(12).* Furthermore, a recent paper has reported that glutaredoxin and thioredoxin (another dithiol enzyme) can serve as efficient electron donors to human plasma GPx *(13).* These results invite speculation that glutaredoxin (or another dithiol enzyme) might reduce the oxidized selenocysteines on plasma GPx at the expense of ascorbic acid, which is abundant in human plasma. The dithiol active site of protein disulfide isomerase is structurally very similar to the glutaredoxin and thioredoxin active sites *(12,14),* and it is secreted by activated platelets *(15).* Protein disulfide isomerase therefore also appears well suited to couple ascorbic acid oxidation to the reduction of hydroperoxides by plasma GPx. Alternatively, it may be envisioned that plasma GPx might get its reducing equivalents from inside cells via a transmembrane thiol-disulfide exchange system. In this regard, it is interesting to note that protein disulfide isomerase *(15,16),* thioredoxin *(17)* and thioredoxin reductase *(18)* have been reported to be present on the outer surfaces of mammalian cell membranes. It is tempting to speculate that our observation that hydrophobic hydroperoxides appear to be more appropriate substrates for plasma GPx may reflect a tendency of plasma GPx to associate with cell membranes. The natural source of reducing equivalents for plasma GPx is an important area for future research that will be key to clarifying the physiological function(s) of this extracellular selenoenzyme.

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REFERENCES

1. G. C. Mills, J. Biol. Chem. **229,** 189-197 (1957).
2. J. W. Forstrom, J. J. Zakowski, and A, L. Tappel, Biochemistry **17,** 2639-2644 (1978).

3. K. Takahashi and H. J. Cohen, *Blood* **68,** 640-645 (1986).
4. K. Takahashi, N. Avissar, J. Whitin, and H. Cohen, *Arch. Biochem. Biophys.* **256,** 677-686 (1987).
5. K. Takahashi, M. Akasaka, Y. Yamamoto, C. Kobayashi, J. Mizoguchi, and J. Koyama, *J. Biochem. (Tokyo)* **108,** 145-148 (1990).
6. L. Flohé, K. D. Aumann, R. Brigelius-Flohé, D. Schomburg, W. Straßburger, and F. Ursini, (1993) in *Active Oxygen, Lipid Peroxides, and Antioxidants,* K. Yagi, ed. CRC, Boca Raton, FL, pp. 299-311.
7. K. R. Maddipati and L. J. Marnett, *J. Biol. Chem.* **262,** 17398-17403 (1987).
8. D. E. Paglia and W. N. Valentine, *J. Lab. Clin. Sci.* **70,** 158-169 (1967).
9. M. M. Bradford, *Anal. Biochem.* **72,** *248* (1976).
10. R. S. Esworthy F. F. Chu, P. Geiger, A. W. Girotti, and J. H. Doroshow, *Arch. Biochem, Biophys.* **307,** *29-34* (1993).
11. Y. Yamamoto and K. Takahashi, *Arch. Biochem. Biophys.* **305,** 541-545 (1993).
22. W. W. Wells, D. P Xu, Y. Yang, and P. A. Rocque, *J. Biol. Chem.* **265,** 15,361-15,364 (1990).
23, M. Bjornstedt, J. Y. Xue, W. H. Huang, B. Akesson, and A. Holmgren, *J. Biol. Chem.* **269,** 29,382-29,384 (1994).
24. J. C. Edman, L. Ellis, R. W. Blacher, R. A. Roth, and W. J. Rutter, *Nature* **317,** 267-270 (1985).
25. K. Chen, T. C. Detwiler, and D. W. Essex, *Br. J. Haematol.* **90,** *425-431* (1995).
26. H. Kroning, T. Kahne, A. Ittenson, A. Franke, and S. Ansorge, *Scand. J. Immunol.* **39,** 346-350 (1994).
17, H. Martin and M. Dean, *Biochem. Biophys. Res. Commun.* **175,** 123-128 (1991).
18. K. U. Schallreuter and J. M. Wood, *Biochim. Biophys. Acta* **967,** 103-109 (1988).